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(54) Title: ELECTROCHEMILUMINESCENT ENZYME BIOSENSORS

(57) Abstract

Electrochemiluminescent enzymes, their preparation and use as biosensors are disclosed. Specifically, two appendages are covalently attached to a desired dehydrogenase enzyme; (1) a nicotinamide adenine cofactor or analog thereof, and (2) a luminescent ruthenium complex. For example, glucose concentration is the following way. A doubly-modified glucose dehydrogenase could oxidize glucose with concomitant reduction of the attached NAD^+ to NADH. Because NADH, but not NAD^+, is able to interact with surface ruthenium to promote ECL, only enzyme molecules that have reacted with glucose will emit light from their ruthenium label in an ECL instrument. The relative close proximity of NADH and ruthenium on the enzyme surface enhances light emission as compared to the same concentrations in free solution. When NADH reduces ruthenium, it returns to become NAD^+, permitting multiple cycles of ECL light emission from a single enzyme molecule. Such biosensors can be used in solution or bound to a solid surface. Assays employing the biosensor molecules can be performed on an IGEN Origen® Analyzer.

SUMMARY OF THE INVENTION

Broadly stated, the invention contemplates an electrochemiluminesence based assay using a biosensor having a dehydrogenase which has been modified to properly position TAG and the desired nucleotide cofactor (coenzyme) near the active site. Such positioning enhances reaction times improves the energy efficiency of the system which improves accuracy, speed and reliability and the like for the detection of enzymes or their substrates and cofactors.

The object of the invention is to both enhance sensitively and specificity of ECL-based assays and to conserve reagent usage.

The monitored or detected analyte is either the substrate of a dehydrogenase or a substance that can be converted into the substrate of a dehydrogenase. The dehydrogenase is the molecule that allows sensing of the analyte by an ECL instrument. It is also envisioned that the biosensor molecule of the invention can be used in conjuction with another enzyme or enzyme system which produces a product which is measured by the biosensor molecule. This would further increase the range of applications.

The dehydrogenase is not present in its natural form, but rather has been chemically-modified so that it has two unnatural appendages. One appendage is a covalently attached functional analog of $NAD(P)^+$, NAD(P)H. Such enzymes have been made (Persson et al. 1991). This nicotinamide cofactor is specifically attached in a way that it can bind in the active site of the enzyme and function as a redox reagent as part of the natural enzyme mechanism. The second appendage is an ECL label such as a derivative of $Ru(bpy)_3^{2+}$. Such proteins have been made (Blackburn et al. 1991).

The way that the biosensor works is as follows:

(1) The analyte is oxidized by the NAD^+ form of the doubly-modified enzyme, causing the enzyme to be converted to the NADH form.

(2) In an ECL instrument, voltage will be applied to the enzyme, causing the NADH appendage to become oxidized by donating an electron to the luminescent ruthenium-containing appendage, which in turn emits light (Downey et al. 1992). As a result, NADH returns to become NAD^+ such that a second molecule of analyte can be oxidized and the cycle can be repeated. The demonstration of electron transfer through ruthenium-labelled proteins has previously been accomplished (Pan et al. 1993; Wuttke et al.)

Some analytes (and their enzymes) that function in the above mechanism are: glucose (glucose dehydrogenase), ethanol (alcohol dehydrogenase), and lactate (lactate dehydrogenase). It is envisioned, however, that other oxidoreductases such as those listed on pages 684-702 of the Enzymes (Academic Press (1979)) by M. Dixon and E. C. Webb could also be utilized. Their selection depends on the location of amino acids near the active site with sidechains which could be linked to either NAD or TAG moities through an appropriate linking group, e.g. ester, thiol ether, etc..

The main advantages of this invention are:

(1) potential high sensitivity and low background. The fixed nature of the reagents (on a single molecule) is desirable because of entropic effects; their effective concentrations relative to each other will be very high which will give substantially better yields (of light) than if they were free in solution.

(2) possibly lowered analysis cost over the same system in which the enzyme, NADH, and the ruthenium compound are free in solution. Because of this, lower concentrations of the reagents will give results equivalent to results obtained with free reagents, and

also because one NADH molecule can be recycled, the cost of analysis will be reduced.

(3) combining three reagents into one molecule is more attractive for immobilized systems. It would be extremely attractive to be able to immobilize biosensors so that they can be reused. If the enzyme alone were immobilized, the other reagents (NADH and $Ru(bpy)_3^{2+}$) would need to be added for each analysis. In this invention, all reagents could be immobilized in a way in which would be functional.

Devices employing biosensor molecules typically employ the molecule in an immobilized state. Immobilization provides stability to the molecule and ensures its continued presence in a desired area of the device for optimal performance. Typical of the techniques used to immobilize the biosensor molecules of this invention are those disclosed on pages 3 through 34 of Methods of Enzymology, Vol. 136 (1987).

At the present time, a preferred use for the glucose dehydrgenase of the invention is as a glucose biosensor. Functional glucose dehydrogenase has been made which has an immobilized NADH molecule (Persson et al., 1991). This modified enzyme could be reacted with $Ru(bpy)_3^{2+}$-NHS ester (IGEN, Inc., Rockville, MD) by conventional means used to attach this compound to surface lysines present on antibodies (Blackburn et al., 1991). Such modification may result in one or more ruthenium appendages (multiple appendages are acceptable or even advantageous if the enzyme remains active). The doubly-modified enzyme could be purified by dialysis or chromatography. Addition of glucose followed by testing in the ECL instrument (conventional analysis for NADH), would give a light signal depending on the activity of the enzyme, which in turn depends on the glucose concentration.

The improvements of the invention over the prior art are set forth above. They include sensitivity, cost, and

ease of immobilization. Higher sensitivity (over comparable assays in which ruthenium and the nicotinamide cofactor are not attached to the dehydrogenase) results from advantages relating to the intramolecular nature of the electron transfer (Pam Liang and Mark Martin, unpublished ECL results). Cost savings will result from the reusable nature of the doubly-modified enzyme (easier recovery as opposed to recovery and reuse of the free enzyme, ruthenium complex, and nicotinamide cofactor). Finally, it would be impractical to separately immobilize all three components on a solid surface in a functional way. Coupling all three into one functional molecule allows solid phase immobilization (and hence convenience and reduce cost, compared to having enzyme, ruthenium, and cofactor free in solution).

Procedures for making NAD-labelled glucose dehydrogenase are known. Since dehydrogenases are a fairly conserved group of enzymes, similar methods could be used with other dehydrogenases. Procedures for labelling proteins (usually antibodies) with $Ru(bpy)_3^{2+}$-NHS ester (through lysins) are also well known (IGEN, Inc. technical notes).

The enzyme biosensor of the invention requires for use an ECL instrument and a solution containing analyte, such as glucose. A solution containing analyte (such as glucose) would be appropriately diluted, if necessary, and mixed in solution with enzyme biosensor. There are at least two ways the analyte could be measured; after fractional enzyme turnover and by rate measurements in the ECL instrument. For the fractional enzyme turnover method the ECL would be measured after a defined time period, less than the time required for all enzyme molecules to complete one turnover. If analyte concentration and time are appropriately adjusted, less than 100% of the enzyme molecules would have reacted with glucose, and less than maximal ECL would be generated. Based on the established kinetic parameters for the

reaction, glucose concentration could be determined. Alternatively, analyte concentration could be determined by rate measurements in the instrument. The enzyme reaction could be carried out at the same time the ECL reaction is in progress (such as by pulsing of voltage), so that NADH would be actively recycled and a rate measurement could be made.

The same system could be used without covalently attaching the reagents. Alternatively, a non-ECL system could be used without ruthenium (free enzyme and free NAD(P)$^+$), where the UV absorbance of generated NAD(P)H could be monitored spectrophotometrically.

Novel NAD(P)H-like compounds could be used that are enzymatically active and also ECL active. Also, non-ruthenium ECL-active lumiphores could be used in place of $Ru(bpy)_3^{2+}$).

DETAILED DESCRIPTION OF THE INVENTION

Mechanism of ECL excitation is as follows. Analyte is oxidized in the presence of the biosensor molecule of the invention which causes the NAD^+ containing appendage to be converted to NADH. $Ru(bpy)_3^{2+}$ (TAG) and NADH are oxidized at the surface of a gold electrode, forming $Ru(bpy)_3^{3+}$ and $NADH^+\cdot$, respectively. (In this description, NAD^+ and TAG are covalently attached to the dehydrogenase.) The $NADH^+\cdot$ spontaneously loses a hydrogen, forming NAD·. The NAD·, a strong reductant, reacts with $Ru(bpy)_3^{3+}$, a strong oxidant, forming the excited state of the detectant, $Ru(bpy)_3^{2+*}$. The excited state decays to the ground state through a normal fluorescence mechanism, emitting a photon having a wavelength of 620 nm.

Organic compounds which are suitable electrochemical detectants include, for example, rubene and 9,10-diphenyl anthracene. Many organometallic compounds are suitable electrochemical detectants, but of preferable use are Ru-containing compounds, such as ruthenium II tris-bipyridine chelate, and Os-containing

compounds. Detectants useful in the presently disclosed invention can be found in U.S. Patent 5,310,687, the contents of which are incorporated herein by reference.

These detectants are stable for long periods. In addition, the detectants are safe and relatively inexpensive. They give a highly characteristic signal and do not occur in nature. Measurements based on luminescence of such detectants are sensitive, fast, reproducible and utilize simple instrumentation. The signal is generated repeatedly by each molecule of the detectant, thereby enhancing the sensitivity with which these detectants may be detected. The preferred electrochemiluminescent detectants of the present invention are conveniently referred to herein as $Ru(bpy)_3^{2+}$. Various amounts of this detectant, or its equivalent, may be employed.

It is also to be noted that these detectants can be used directly in biological or food samples without pretreatment of sample.

The energy necessary for formation of the excited state arises from the large difference in electrochemical potentials of the $Ru(bpy)_3^{3+}$ and NAD·. The excited-state $Ru(bpy)_3^{2+*}$ decays through a normal fluorescence mechanism, emitting a photon at 620 nm. This process regenerates the original form of the $Ru(bpy)_3^{2+}$, which is free to cycle multiple times through the reaction sequence. Each ECL-active detectant, therefore, can emit many photons during each measurement cycle, thereby enhancing detection.

Quantification of the $Ru(bpy)_3^{2+}$ detectant can be readily automated with relatively uncomplicated instrumentation. The heart of an instrument is the electrochemical flow-cell, containing the working electrodes and counter electrodes for initiation of the ECL reaction. Both of the electrodes are fabricated from gold, but other materials have been used with various degrees of success. A potentiostat applies various

outside of the active site will be acetimidylated by adding 2.1 M ethyl acetimidate-HCl (Sigma Chem. Co.) (volume will be added to increase enzyme solution by 5%). (Stock solution of 2.1 M ethyl acetimidate will be made up fresh and pH adjusted to 8.0). The reaction will be allowed to go for one hour at 25° C, then three more additions of ethyl acetimidate will be made hourly (total reaction time = 4 hours). The protein will then be dialyzed extensively, against 33 mM sodium phosphate, 0.5 mM EDTA, 2.0 mM adenosine monophosphate, pH 8.0, then against 0.2 M sodium bicarbonate, pH 8.0. The external lysine-protected NAD-ADH will be reacted with an NHS ester derivative of $Ru(bpy)_3^{2+}$ (IGEN, Inc., Gaithersburg, MD) by established means. Unreacted (free) $Ru(bpy)_3^{2+}$ will be removed by dialysis against a neutral buffer. By comparison with literature reports only one $Ru(bpy)_3^{2+}$ per enzyme subunit will be incorporated, on Lys 228 (See Brändén et al., Experientia Supplemental 36, J. Jeffrey, ed., Dehydrogenases, pp. 62-3.).

Example 2: Assays of NAD^+-ADH-$Ru(bpy)_3^{2+}$ Catalytic Activity

Assays 1 and 2 are based on M.-O. Månsson, et al., Eur. J. Biochem. 86, 455-463 (1978). Assay 3 is the electrochemiluminescent assay in which enzymatic conversion of ethanol by the NAD^+-ADH-$Ru(bpy)_3^{2+}$ conjugate is accompanied by light emission.

ethanol. The absorbance of the mixture is continuously read in a spectrophotometer at 490 nm.

Assay 3: Electrochemiluminescent Assay

The assay is performed similarly to Assay 1 (above). However, instead of measuring the absorbance increase when ethanol is added, similar enzyme conjugate solutions (with and without added ethanol) will be measured in an IGEN ECL Analyzer (IGEN, Inc., Gaithersburg, MD). In the absence of ethanol the enzyme conjugate will be in the form NAD^+-ADH-$Ru(bpy)_3^{2+}$ (non-electrochemiluminescent). Following enzymatic conversion of ethanol to acetaldehyde, the enzyme conjugate will be in the form NADH-ADH-$Ru(bpy)_3^{2+}$ (electrochemiluminescent). Moreover, voltage is applied to the enzyme conjugate in the ECL instrument, light is emitted, and the conjugate returns to the original form (NAD^+-ADH-$Ru(bpy)_3^{2+}$). This original form can then catalyze oxidation of another molecule of ethanol, which would convert the enzyme conjugate once against to the electrochemiluminescent NADH form. Thus, multiple photons can be generated by the enzyme conjugate in the presence of ethanol.

Example 3: Preparation of a NAD^+-Mutant Glucose Dehydrogenase-$Ru(bpy)_3^{2+}$

In this example, a glucose dehydrogenase mutant will be prepared to contain a strategically-located surface sulfhydryl group which can react with an NAD^+ analog to produce an NAD^+-enzyme conjugate. The mutation is

positioned so that the tethered NAD^+ molecule can bind to the NAD^+ binding site in the enzyme and be enzymatically efficiently reduced to NADH. The mutant glucose dehydrogenase-NAD^+ conjugate will then be reacted with an N-hydroxysuccinimide (NSH) derivative of $Ru(bpy)_3^{2+}$ to yield a doubly-modified enzyme; NAD^+-GlcDH-$Ru(bpy)_3^{2+}$. This enzyme conjugate will be luminescent in an ECL instrument. For every glucose molecule that the enzyme catalyzes, the surface NAD^+ will be converted to NADH. In an ECL instrument (IGEN, Inc., Gaithersburg, MD), NADH but not NAD^+ will cause enzyme surface-immobilized $Ru(bpy)_3^{2+}$ to emit a photon of light. Thus, a molecule of glucose will result in a photon of light to be emitted by the doubly-modified enzyme. Moreover, in the ECL process, NADH is reconverted to NAD^+. Thus, the doubly-modified enzyme is regenerated by emitting light and can be used repeatedly.

Part 1: Preparation of the Mutant GlcDH.

Mutant GlcDH has been previously prepared by site-directed mutagenesis (M. Persson, et al., Bio/Technology (1991) 9,280-284). The residue asp^{44} in glucose dehydrogenase was mutated to a cys^{44} by standard mutagenesis protocol. The mutant protein (GlcDHcys^{44}) was expressed in E. coli and purified by conventional means.

Part 2: Preparation of a Cysteine-Reactive NAD^+ Derivative.

A thiol reactive NAD^+ analog has been prepared (M. Persson, et al., Bio/Technology (1991) 9, 280-284). Essentially, the method involves reaction of two commercially-available reagents; N-succinimidyl-3-[2-pyridyldithio]propionate (SpDP; Pierce Chem. Co.) and N^6[6-aminohexyl-(carbamoylmethyl)-NAD (Sigma Chem. Co.). The resulting product will react with the $GlcDHcys^{44}$ to yield the desired NAD^+-modified enzyme. Such an NAD^+-labelled GlcDH has been prepared (M. Persson, et al., Bio/Technology (1991) 9, 280-284).

Part 3: Preparation of NAD^+-$GlcDHcys^{44}$-$Ru(bpy)_3^{2+}$

The NAD^+-$GlcDHcys^{44}$ molecule prepared in Part 2 will be reacted with an NHS ester derivative of $Ru(bpy)_3^{2+}$ (IGEN, Inc.) using established protocols (0.2 M $NaHCO_3$, pH 8.0, room temperature) for reactions of this reagent with proteins (IGEN technical notes). One or more lysine residues on the surface of NAD^+-$GlcDHcys^{44}$ will be covalently linked to $Ru(bpy)_3^{2+}$ as a result of the reaction. Following the reaction, free unreacted $Ru(bpy)_3^{2+}$ will be removed by dialysis to yield NAD^+-$GlcDHcys^{44}$-$Ru(bpy)_3^{2+}$.

Example 4: ECL Detection of Glucose Using NAD^+-$GlcDHcys^{44}$-$Ru(bpy)_3^{2+}$

NAD^+-$GlcDHcys^{44}$-$Ru(bpy)_3^{2+}$ will concurrently oxidize glucose to gluconolactone and reduce immobilized NAD^+ to

NADH. Next, in an ECL instrument (IGEN, Inc., Gaithersburg, MD), enzyme-immobilized NADH will efficiently cause neighboring immobilized $Ru(bpy)_3^{2+}$ to emit light. Thus, the doubly-modified enzyme will report the presence of glucose by emitting light.

In the test tube, enzyme (NAD^+-GlcDHcys^{44}-$Ru(bpy)_3^{2+}$) will be added (≤1 μM) to 0.1 M sodium phosphate buffer (pH 7.2). The solution will be equilibrated at 25.0 ± 0.1°C. A glucose-containing solution is added in a small volume. In the absence of glucose the enzyme conjugate will be in the form NAD^+-GlcDHcys^{44} -$Ru(bpy)_3^{2+}$ (non-electrochemiluminescent). Following enzymatic conversion of glucose to gluconolactone, the enzyme conjugate will be in the form NADH-GlcDHcys^{44}-$Ru(bpy)_3^{2+}$ (electrochemiluminescent). Moreover, when voltage will be applied to the enzyme conjugate in the ECL instrument, light is emitted, and the conjugate returns to the original form (NAD^+-GlcDHcys^{44}-$Ru(bpy)_3^{2+}$). This original form can then catalyze oxidation of another molecule of glucose, which would convert the enzyme conjugate once again to the electrochemiluminescent NADH form. Thus, multiple photons can be generated by the enzyme conjugate in the presence of glucose.

Although the examples illustrate various modifications of the present invention, other variations will suggest themselves to those skilled in the art in light of the above disclosure. It is to be understood,

WHAT IS CLAIMED IS:

1. An oxidoreductase conjugate capable of generating a chemiluminescent signal comprising a co-factor and a TAG moiety separately linked in close proximity to an active site of the dehydrogenase in a manner which permits their electrochemical interaction with each other and a substrate contained within the active site.

2. The oxidoreductase conjugate of claim 1 wherein oxidoreductase is a dehydrogenase is selected from the group consisting of glucose dehydrogenase, alcohol dehydrogenase and lactate dehydrogenase.

3. The dehydrogenase conjugate of claim 2 wherein the dehydrogenase is glucose dehydrogenase.

4. The dehydrogenase conjugate of claim 3 wherein the co-factor is NADH and the TAG is $Ru(bpy)_3^{2+}$.

5. The dehydrogenase conjugate of claim 3 wherein the TAG is covalently attached to a lysine located near the active site.

6. The dehydrogenase conjugate of claim 3 wherein the co-factor is covalently linked to a cysteine located near the active site.

7. A biosensor comprising an oxidoreductase conjugate of claim 1.

8. The biosensor of claim 7 wherein the oxidoreductase is functionally affixed to an electrode.

9. The biosensor of claim 7 wherein the oxidoreductase is immobilized.

10. The biosensor of claim 7 further comprises an electrode which is located in close proximity to the oxidoreductase conjugate to permit the optimal inducement of TAG to generate a luminescent signal.

11. The biosensor of claim 10 wherein the oxidoreductase conjugate is immobilized to the electrode.

12. A method for the determination of the concentration of an enzyme substrate in a specimen comprising the steps of:

(a) contacting said specimen containing said enzyme substrate with the oxidoreductase conjugate of claim 1 under conditions which permit the oxidation of the enzyme substrate;

(b) measuring the change in chemiluminescent from a base reading; and